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Universidade Estadual de Londrina
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Morotti, Fábio; Tadeu Campos, Jefferson; Marcondes Seneda, Marcelo
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Universidade Estadual de Londrina
Londrina, Brasil

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Fixed-time artificial insemination using injectable progesterone: ovarian follicular dynamics and pregnancy rates of Nelore cows (*Bos indicus*) with and without a corpus luteum

Inseminação artificial em tempo fixo utilizando progesterona injetável: dinâmica folicular ovariana e taxas de prenhez de vacas Nelore (*Bos indicus*) com e sem um corpo lúteo

Fábio Morotti¹; Jefferson Tadeu Campos¹; Marcelo Marcondes Seneda^{2*}

Abstract

This study evaluated the follicular dynamics and the pregnancy rates of Nelore cows with or without a CL synchronized with FTAI employing injectable progesterone (P4). Multiparous cows (n=44), 45-65 days postpartum were allocated into a 2x2 factorial. Eleven cows with and 11 without a CL received a conventional protocol of FTAI (D0/1 g P4 + 2 mg EB; D8/500 µg cloprostenol, 300 IU eCG and 24 h later 1 mg EB; D10/FTAI). Other 11 cows with and 11 without a CL received 350 mg of injectable P4 (300 mg sc and 50 mg im) and 2 mg of EB im on D0. On D6, 500 µg of cloprostenol and 300 IU of eCG were administered im. After 24 h, 1 mg of EB was administered im and FTAI was performed on D8. The results were analyzed by ANOVA and Tukey or by Chi-square test ($p \leq 0.05$). The presence of follicles ≥ 5 mm on D4, follicular growth rate and CL diameter were similar between groups (50-55%, 1.1-1.2 mm/day and 16.1-17.6 mm; respectively). The diameter of the dominant follicle (DF) at FTAI and ovulatory follicle (OF) were similar between groups with CL (10.5 \pm 1.8 and 11.4 \pm 1.3 mm, respectively) and without CL (10.8 \pm 2.3 and 11.1 \pm 2.2 mm, respectively) but device was higher than injectable (11.7 \pm 2.0 and 11.8 \pm 1.7 vs. 9.7 \pm 1.7 and 10.6 \pm 2.0 mm, respectively). The ovulation and pregnancy rate were similar between groups with CL (59% and 36%, respectively) and without CL (77% and 27%, respectively) but device was higher than injectable (91% and 45% vs. 45.5% and 18%; respectively). The ciclicity did not affect the follicular dynamics and pregnancy rate. The device showed higher diameter of the DF at FTAI, OF and ovulation and pregnancy rates. However, the injectable P4 produced similar percentages of follicles ≥ 5 mm on D4, follicular growth rate and CL diameter, raising the possibility of further investigation.

Key words: *Bos indicus*, ultrasonography, follicular growth, ovulation and dominant follicle

Resumo

Este estudo avaliou a dinâmica folicular e a taxa de prenhez de vacas Nelore, com ou sem CL sincronizadas com protocolo de IATF empregando progesterona injetável (P4). Vacas multíparas (n=44), 45-65 dias pós-parto foram alocados em um fatorial 2x2. Onze vacas com e 11 sem CL receberam um protocolo convencional de IATF (D0/1 g P4 + 2 mg BE; D8/500 µg cloprostenol, 300 UI eCG e 24 h depois 1 mg BE; D10/IATF). Outras 11 vacas com e 11 sem CL receberam 350 mg de P4 injetável (300 mg SC e 50 mg IM) e 2 mg de BE IM no D0. No D6, 500 µg de cloprostenol e 300 UI de eCG foram administradas IM.

¹ Discentes de Doutorado do Programa de Pós-Graduação em Ciência Animal, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: fabiomorotti@hotmail.com; jeffersomcampos@seromtel.com.br

² Prof. Dr. do Deptº de Clínicas Veterinárias, Centro de Ciências Agrárias, UEL, Londrina, PR, Brasil. E-mail: mseneda@uel.br

* Author for correspondence

Após 24 h, 1 mg de BE foi administrado IM e IATF foi realizada no D8. Os resultados foram analisados por ANOVA e teste de Tukey ou Qui-quadrado ($p \leq 0,05$). A presença de folículos ≥ 5 mm no D4, a taxa de crescimento folicular e o diâmetro do CL foram semelhantes entre os grupos (50-55%, 1,1-1,2mm/dia e 16,1-17,6mm, respectivamente). O diâmetro do folículo dominante (FD) na IATF e do folículo ovulatório (FO) foram semelhantes entre os grupos com CL ($10,5 \pm 1,8$ e $11,4 \pm 1,3$ mm, respectivamente) e sem CL ($10,8 \pm 2,3$ e $11,1 \pm 2,2$ mm, respectivamente), mas foi maior no grupo dispositivo comparado ao injetável ($11,7 \pm 2,0$ e $11,8 \pm 1,7$ vs. $9,7 \pm 1,7$ e $10,6 \pm 2,0$ mm, respectivamente). A taxa de ovulação e de prenhez foram semelhantes entre os grupos com CL (59% e 36%, respectivamente) e sem CL (77% e 27%, respectivamente), mas foi mais elevada no grupo dispositivo comparado com o injetável (91% e 45% vs. 45,5% e 18%, respectivamente). A ciclicidade não interferiu na dinâmica folicular e na taxa de gestação. O dispositivo apresentou maior diâmetro do FD na IATF, do FO e das taxas de ovulação e prenhez. No entanto, a P4 injetável produziu percentagens semelhantes de folículos ≥ 5 mm no D4, de taxa de crescimento folicular e diâmetro do CL, levantando a possibilidade de novas investigações.

Palavras-chave: *Bos indicus*, ultrassonografia, crescimento folicular, ovulação e folículo dominante

Introduction

Brazil has a privileged position in the field of artificial insemination (AI) of cattle, having the largest commercial herd (200 million cattle) and the largest market for fixed-time artificial insemination (FTAI) in the world. Recently, the use of AI showed impressive growth and this progress was mainly due to the increased use of FTAI. In 2002, approximately 100,000 cows were inseminated using FTAI and in 2012, this number was more than 6.5 million.

Progesterone (P4) or progestin is the principal hormonal basis of estrus synchronization in most FTAI programs for cattle. This hormone can be used in the form of an intravaginal device, an ear implant or an oral formulation (BARUSELLI et al., 2004a).

During treatment using the intravaginal device (5-10 days), P4 exerts negative feedback on estradiol and inhibits the LH surge, thus preventing estrus and the ovulation of the dominant follicle (DF) (BARUSELLI et al., 2004a). When associated with estrogen on D0, P4 promotes the regression of the DF and the synchronized emergence of a new follicular wave. After this treatment, PGF2 α can be administered to promote estrus synchronization, and estradiol, GnRH or LH can be administered to induce ovulation synchronization (BÓ et al., 2002).

Currently, there are many protocols of estradiol/P4-based FTAI that efficiently control the estrus and ovulation of cattle (SÁ FILHO et al., 2010;

SALES et al., 2012) but to improve the reproductive management, minimize service and reduce costs, it is necessary to remain at the forefront of advances in reproductive biotechnology. The use of an injectable P4 source to control the estrous cycle would more practical the current FTAI programs and other reproductive biotechnologies because one of the major advantages of injectable P4 is the labor reduction when FTAI is used on large scale, due mainly the facility of application route.

The effects of injectable P4 on controlling the estrous cycle of cattle (ULBERG; LINDLEY, 1960; FIKE et al., 1999) and in FTAI protocols (Morotti et al., unpublished) has been scarcely studied. Therefore, the aim of this study was to evaluate ovarian follicular dynamics and the pregnancy rates of Nelore cows (*Bos indicus*), with or without CL, subjected to FTAI using injectable P4 or an intravaginal P4 device.

Materials and Methods

Site, animals and feed management

The experiment was conducted during the breeding season (November to March) in South America, latitude 23°24'23" and longitude 57°26'4". The climate in this region is tropical, with an average temperature of 24°C, and a rainy season from November to January.

Forty-four, multiparous Nelore cows (*Bos indicus*) between 72 and 84 months of age and at 45 to 65 days postpartum were used in this study. The animals had body condition scores (BCSs) of between 2.5 and 3.5 (2.8 ± 0.1) on a scale of 0 to 5 (AYRES et al., 2009) and an average weight of 410.3 ± 47.5 kg.

During the experimental period, the cows were maintained by continuous grazing of *Brachiaria brizantha* and *Brachiaria humindícula*. They were given access to mineralized salt and water *ad libitum*.

Animal selection

The cows were previously selected according to their ovarian activity. Evaluations by palpation and transrectal ultrasonography, using an Aloka model SSD-500 ultrasound instrument equipped with a 5 MHz linear transducer (Aloka, Tokyo, Japan), were performed to select 22 cows with a CL and 22 cows without a CL and with large follicles < 8 mm in diameter (BARUSELLI et al., 2004a).

Experimental design and treatments

The selected animals were allocated into a 2x2 factorial matrix. Eleven cows with and 11 cows without a CL were subjected to conventional FTAI using an intravaginal device containing 1 g of P4 (DIB®, Syntex, Buenos Aires, Argentina) and an intramuscular (im) application of 2 mg estradiol benzoate (EB) (Syntex®, Syntex, Buenos Aires, Argentina) on D0. Eight days later (D8), the device was removed and 500 µg of cloprostenol (DL Cyclase®, Syntex, Buenos Aires, Argentina) and 300 IU of equine chorionic gonadotropin (eCG); (Novormon®, Syntex, Buenos Aires, Argentina) were administered im. On D9, 1 mg of EB (Syntex®, Syntex, Buenos Aires, Argentina) was administered im and FTAI performed on D10, 48 h after device removal.

The other cows (11 with and 11 without CL) received 350 mg of injectable P4 (300 mg subcutaneously³ and 50 mg im⁴) and 2 mg of EB (im) on D0. Six days later (D6), 500 µg of cloprostenol and 300 IU of eCG were administered im. On D7, 1 mg of EB im was administered, and FTAI was performed on D8, 24 h after the ovulation inductor. This timing was established based on the results of previous studies (Unpublished data).

Follicular dynamics

The follicular dynamics were evaluated using transvaginal ultrasonography with an Aloka® SSD-500 ultrasound system equipped with a 7.5 MHz micro convex transducer (Aloka, Tokyo, Japan) and coupled to a follicular aspiration guide.

Evaluations were performed on D0, D4 and every 24 h thereafter until FTAI. Ovulation was monitored every 12 h after insemination, and the CL was assessed 12 days after FTAI. All of the data were obtained for both ovaries and recorded individually for further monitoring.

For each evaluation, both ovaries were ultrasonically scanned, and the follicles ≥ 5 mm were identified and measured using the ultrasonography package. The average of two cross-sectional linear measurements of the follicular antrum was calculated (GINTHER; KNOPF; KASTELIC, 1989).

Behavioral estrus was observed twice daily for 1 h from 6:00 a.m. to 7:00 p.m. The observations began at the time of cloprostenol administration (D6 for injectable P4 and D8 for the intravaginal device) and ended 60 h afterward. Estrus was considered to have occurred when the cow stood to be mounted by another cow (standing estrus).

The DF was defined as the follicle that grew to at least > 8 mm and exceeded the diameter of

³ Natural progesterone in vehicle sesame and peanut oil (slow absorption).

⁴ Natural progesterone in vehicle sesame and peanut oil (fast absorption).

all other follicles (FIGUEIREDO et al., 1997). Ovulation was detected by the absence of the previously identified DF and confirmed by the later presence of a CL in the same ovary. On the 12th day after FTAI, the CL was measured in the ovary that previously contained the DF (GINTHER; KNOPF; KASTELIC, 1989). The pregnancy rate was determined by ultrasonography 35 days after the FTAI.

Parameters evaluated and statistical analyses

The animals were evaluated for the presence of follicles ≥ 5 mm on D4 (%), the average rate of follicular growth (mm/day), the rate of behavioral estrus (%), the diameter of the DF at FTAI (mm), the diameter of the ovulatory follicle (OF) (mm), the ovulation rate (%), the diameter of the CL (mm) and the pregnancy rate (%)

Parametric data were assessed using an analysis of variance (ANOVA). If the difference between the groups was significant, the data were examined using Tukey test. Frequency data and dichotomous variables were analyzed using the Chi-squared test.

All of the data were analyzed using the Statistical Software MNITAB16 program. The significance

level for rejecting the H_0 (null hypothesis) was 5%; namely, a level of significance ≤ 0.05 was considered to indicate an effect of the categorical variables and their interactions. The parametric data are presented as the mean values \pm one standard deviation ($M \pm SD$).

Results and Discussion

To our knowledge, this is the first study evaluating the use of injectable P4 (with a single parenteral application) for FTAI of cattle. We described the protocol used and the first results concerning the follicular dynamics in Nelore cows with and without CL. This study is expected to serve as the basis for the use of injectable P4, considering the lack of information about this product.

The percent of follicles ≥ 5 mm on D4, follicular growth, estrous behavior rate and diameter of the CL were similar for the cows that were synchronized using an intravaginal device or injectable P4. However, the animals that received the injectable P4 exhibited a DF with a smaller diameter at FTAI, an OF with a smaller diameter and lower ovulation and pregnancy rates (Table 1).

Table 1. Follicular dynamics data and pregnancy rates of Nelore cows, with or without CL, synchronized using a protocol based on injectable P4 or an intravaginal device.

Treatments	Follicles ≥ 5 mm on D4 (%/mm)	Follicular growth (mm/day)	Estrous behavior (%)	Diameter of the DF at FTAI (mm)	Diameter of the OF (mm)	Ovulation rate (%)	Diameter of the CL (mm)	Pregnancy rate (%)
Corpus luteum	<i>Present</i>	49.9 6.1 \pm 0.8	1.14 \pm 0.3	77.2	10.5 \pm 1.8	11.4 \pm 1.3	59.0	17.3 \pm 3.8 36.3
	<i>Absent</i>	54.5 6.6 \pm 0.8	1.15 \pm 0.4	86.3	10.8 \pm 2.3	11.1 \pm 2.2	77.2	16.6 \pm 3.2 27.2
	<i>p-value</i>	0.165	0.942	0.434	0.705	0.563	0.195	0.489 0.517
Progesterone	<i>Intravaginal</i>	49.9 6.2 \pm 0.8	1.10 \pm 0.3	81.2	11.7 \pm 2.0 ^a	11.8 \pm 1.7 ^a	90.9 ^a	17.6 \pm 3.5 45.4 ^a
	<i>Injectable</i>	54.5 6.5 \pm 0.9	1.19 \pm 0.4	81.2	9.7 \pm 1.7 ^b	10.6 \pm 1.8 ^b	45.5 ^b	16.1 \pm 3.4 18.1 ^b
	<i>p-value</i>	0.376	0.403	1.00	0.003*	0.005*	0.001*	0.211 0.050*
CL vs. P4	<i>Mean</i>	6.4 \pm 0.8	1.14 \pm 0.3	81.5.2	10.7 \pm 2.1	11.3 \pm 1.8	68.1	17.0 \pm 3.4 31.8
	<i>Interaction</i>	0.958	0.301	0.748	0.564	0.033* ¹	0.007* ²	0.594 0.203

* Significant difference ($p < 0.05$). Different letters (a, b) within a column indicate that the values are significantly different ($p < 0.05$) for the interactions among all of the factors. (¹, ²) For the effect of injectable P4 and the intravaginal device on the diameter of the OF and the ovulation rate of cows with or without the CL, refer to the Table 2.

Source: Elaboration of the authors.

The average rate of follicular growth in our study was similar in the groups that received the intravaginal device (1.10 ± 0.3 mm/day) or the injectable P4 (1.19 ± 0.4 mm/day; $p=0.403$). No interaction was observed between the ovarian activity and the form of P4 used; namely, the rates of follicular growth in the cows treated with injectable P4 or with the intravaginal device, regardless of the presence or absence of CL, were similar. These rates are similar to those obtained by Coutinho et al. (2007) (1.0 ± 0.1 mm/day) in multiparous *Bos indicus* cows and higher than those observed by Callejas et al. (2006) in *Bos taurus* cows treated with the following daily doses of P4: 600 mg (0.1 ± 0.1 mm/day), 400 mg (0.6 ± 0.2 mm/day) or 200 mg (0.5 ± 0.1 mm/day). In this study, the dose of injectable P4 (350 mg P4 only on D0) chosen induced follicular growth rates similar to those induced using the intravaginal device.

The cows synchronized using the intravaginal device showed a larger diameter of the DF at FTAI (11.7 ± 2.0 mm) compared with those receiving injectable P4 (9.7 ± 1.7 mm; $p=0.003$). However, no interaction between the ovarian activity and the form of P4 used was observed. In *Bos indicus* beef cows synchronized using an estradiol/P4-based FTAI protocol, Sá Filho et al. (2010) found an average diameter of 11.1 ± 0.3 mm at FTAI. Siqueira et al. (2009) also reported similar data in

Bos taurus beef cows, which displayed follicular diameters of 10.5 ± 0.1 , 10.7 ± 0.1 and 10.6 ± 0.4 mm when synchronized using an intravaginal device containing 250 mg of medroxyprogesterone acetate, an intravaginal device containing 250 mg of medroxyprogesterone acetate plus 100 mg of P4 given im or an intravaginal device containing 1 g of P4, respectively.

The cows synchronized using the intravaginal device displayed a larger diameter of the OF (11.8 ± 1.7 mm) than did the cows that received injectable P4 (10.6 ± 1.8 mm; $p=0.005$). For this variable, the association of the factors of the intravaginal device and the absence of the CL provided a larger diameter of the OF (12.4 ± 1.3 mm) than did the factors of the injectable P4 and the absence of the CL (10 ± 2.0 mm; $p=0.033$). In contrast, the presence of the CL in cows treated with injectable P4 (12.2 ± 1.4 mm) yielded results similar to those that received the intravaginal device (12.1 ± 1.2 mm; $p>0.05$); (Table 2). In postpartum beef cows, the OF size can vary widely and may be < 11 mm or > 16 mm [13]. The data obtained in the present study (intravaginal and injectable groups) showed smaller diameters of the OF than were observed by Sales et al. (2012) in Nelore cows undergoing FTAI, which were 13.1 ± 0.4 and 13.9 ± 0.4 mm for ovulation induced by EB or EC, respectively.

Table 2. Average diameter of the ovulatory follicle and average ovulation rate of Nelore cows with or without CL, using a protocol based on injectable P4 or an intravaginal device.

Progesterone vs. Corpus luteum	Ovulatory follicle ¹ (mm)	Ovulation rate ² (%)
Injectable P4 with CL	12.2 ± 1.4^a	36.3 ^c
Injectable P4 without CL	10 ± 2.0^b	54.5 ^{bc}
Intravaginal device with CL	12.1 ± 1.2^a	81.8 ^{ab}
Intravaginal device without CL	12.4 ± 1.3^a	100 ^a

Different letters (a, b, c) within a column indicate that the values are significantly different ($p < 0.05$).

Source: Elaboration of the authors.

The ovulation rate was lower in the animals treated with injectable P4 (45.5%, 10/22) than

in those synchronized using the intravaginal device (90.9%, 20/22; $p=0.001$). Furthermore, an

interaction between ovarian activity and P4 use was observed ($p=0.007$). Cows without a CL that received an intravaginal device exhibited a better ovulation rate (100%, 11/11) than those treated with injectable P4, whether without a CL (54.5%, 6/11) or with a CL (36.3%, 4/11). However, cows without a CL that were synchronized using injectable P4 had an ovulation rate similar to that of the cows with a CL that were treated using the intravaginal device (81.8%, 9/11) (Table 2). Most of the cows ovulated within 12-24 h of FTAI (90% in the injectable P4 group and 95% in the intravaginal device group). In Nelore cows that were synchronized using an estradiol/P4-based FTAI protocol, Sales et al. (2012) observed a lower EB-induced ovulation rate with the use of the intravaginal device of 77.8% of the cows (21/27). The ovulation rate of the injectable P4 group was lower than that observed in the control group or those reported in the literature.

Generally, estradiol/P4-based FTAI protocols are highly efficient in synchronizing a wave of follicular growth and ovulation in *Bos indicus* cows (SÁ FILHO et al., 2011; SALES et al., 2012). In the present study, only one cow with an intravaginal device showed no follicular recruitment or growth, whereas 95% of the cows responded to the protocol and 90.9% ovulated. All of the cows given the injectable P4 demonstrated follicular recruitment and growth, with follicles > 7 mm in diameter at FTAI; however, only 45.4% of this group ovulated.

Other than the individual characteristics of P4 metabolism, it is possible that the BCS also had some influence (RODRIGUES et al., 2011), resulting in variable levels of LH and affecting ovulation. In the present study, the ovulation rate was higher in cows with a BCS close to 3.

In the animals that received the injectable P4, ultrasonography performed until 48 h after FTAI revealed that some of the non-ovulatory cows continued to experience DF growth, followed by a reduction in the DF diameter without achieving ovulation. A longer period of DF growth might

increase the diameter of the OF and the ovulation rate. Other ovulation inducers, such as GnRH and LH, could also be used. A study of the plasma profile of associated with injectable P4 may yield important information to better direct its use in FTAI protocols.

An interesting result of this study is that the CL size was similar in the groups, regardless of their cyclicity (CL present: 17.3 ± 3.8 mm and CL absent: 16.6 ± 3.2 mm; $p=0.489$) or the form of P4 utilized (intravaginal device: 17.6 ± 3.5 mm and injectable P4: 16.1 ± 3.4 mm; $p=0.211$). This result suggests the possibility of using injectable P4 in the protocol for fixed-timed embryo transfer research.

In our study, the small number of animals ($n=44$) is a limitation in evaluating the pregnancy rate; nevertheless, we considered that it was important to provide these data due lack of information of injectable P4. The pregnancy rate was higher in the cows synchronized using the intravaginal device (45.4%, 10/22) compared than those treated with injectable P4 (18.1%, 4/22; $p=0.05$). A similar rate was found in beef cows treated with 50 mg of P4 daily (for 14 days), followed 72 h later by 0.5 mg of EB, which had a 15% (4/26) pregnancy rate. In the animals that received 25 mg of P4 and 1 mg of EB, the pregnancy rate was 38% (16/42) (ULBERG; LINDLEY, 1960). The lower fertility observed in our study was due to the lower ovulation rate of the animals that received the injectable P4. Despite the effect of P4 on the pregnancy rate, an interaction between ovarian activity and P4 use was not observed.

The pregnancy rates of the intravaginal device groups were lower than those found by Bader et al. (2005) and Schafer et al. (2007), but similar to those found by Colazo et al. (2004). In a study of Nelore cows synchronized using an intravaginal P4 device, Silveira et al. (2012) reported pregnancy rates of 53%. In contrast, Baruselli et al. (2004b) obtained a 43% rate and reported that these variations are attributable to different types of

property management, thereby allowing diversity in the pregnancy rate from 40 to 60%.

In conclusion, Nelore cows treated with an intravaginal device and subjected to a FTAI protocol exhibited DFs with a larger diameter at FTAI, OFs of a larger diameter, and higher ovulation and pregnancy rates. However, the cows that received injectable P4 had a similar percentage of follicles ≥ 5 mm on D4, rate of follicular growth, rate of behavioral estrus and a CL diameter, raising the possibility of further investigation. It is possible that the injectable P4 is not completely metabolized during the treatment for FTAI, resulting in variable levels of LH and blocking ovulation.

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